ENDOCRINE STUDIES IN ACNE VULGARIS

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SYNOPSIS

The frequent occurrence of acne vulgaris in association with hyperadrenocorticism raised the question as to whether all cases of acne are accompanied by some abnormality of adrenal function. Previous work on the endocrine status in acne has indicated that an excess of androgen over estrogen is characteristic of the disease. It was postulated that the origin of this disequilibrium was the adrenal gland. Accordingly an exploratory study was made on a small group of acne patients to see if abnormal adrenal responses could be detected. This involved cortin, 17-ketosteroids and estrogen estimations on three consecutive twenty-four hour urines. On the second day each subject received 25 mg. ACTH intravenously over six to seven hours. An investigation of a fluorometric method of urinary estrogen estimation was carried on at the same time, but the defects revealed were such that the results are of no practical significance.

It was found that 17-ketosteroid values gave the most information and significant differences between acne, and control groups were found in females on the first day of the test and in males on the second day. These limited differences do not justify the conclusion that acne is due to mild adrenal overactivity until further studies have been done. For these it may be necessary to include improved estrogen estimations.

THE UNIVERSITY OF ALBERTA

ENDOCRINE STUDIES IN ACNE VULGARIS

A DISSERTATION

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

FACULTY OF ARTS AND SCIENCE

by

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EDMONTON, ALBERTA

SEPTEMBER, 1953.

ACKNLOWEDGEMENT

This investigation was carried out with the assistance of grants from the National Research Council and the John and Mary R. Markle Foundation under the direction of Dr. D. R. Wilson, to whom the writer wishes to acknowledge his indebtedness. He would like to express his appreciation for the kind co-operation of Dr. Paul Rentiers and the late Dr. Harold Orr in making clinical material available. The writer is also indebted to Dr. R. E. Bell for valuable advice and criticism, and to Miss Doris Morison for technical assistance.

The provision of samples of crystalline estrogens by Dr. Fred Wrigley, Ciba Company, and L. A. Sweet, Parke, Davis & Company is gratefully acknowledged.

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INTRODUCTION

Acne vulgaris is defined as "a chronic inflammatory disease of the pilosebaceous follicles characterized by comedones, papules, pustules, cysts, and nodules." (1).

The term pilosebaceous follicle or apparatus in dermatological usage refers to the hair follicle and its attendant sebaceous gland or glands whose excretory ducts open into the neck of the follicle.

Certain histological features should be noted as having a bearing on the pathogenesis of the disease. The lining of the follicle from the level of the duct openings to the surface of the skin is continuous with the epidermis. The glands themselves consist of rounded sacs enclosed by a basement membrance and a single layer of thin cells. More centrally the cells are larger and contain fat droplets while towards the lumen of the duct cells have broken down into fatty detritus which constitutes the secretion of the gland (2).

The primary lesion in acne vulgaris is the comedo or blackhead consisting of a follicle plugged by masses of sebaceous material. One of the factors in its formation is thought to be hyperkeratosis of the mouth and neck of the follicle which tends to obstruct the orifice. The histologic and bacteriologic studies of Lynch (3) do not indicate that infection plays a part in the formation of the comedo but that inflammation is a usual complication leading to the formation of papules, pustules, cysts and nodules.

The view that inflammation is a primary factor and that it is due to specific acne-causing organisms is not now generally accepted. The suggestion has been made (4) in view of the occurrence of giant cells in the inflammatory exudate that it represents a type of foreign body reaction to retained comedo lipid or its breakdown products. Whatever the cause of the inflammatory lesions any explanation of the pathogenesis of acne must first account for comedo formation which precedes the other lesions.

Acne is regarded as one of the most common skin diseases in adolescence (5). In Bloch's report (6) of an examination of 4191 boys and girls between the ages of 6 and 19 he found acne in the broadest sense (i.e. all cases down to a few comedones) in 2681 of the sample, about 64%. True clinical acne was found in 8% of the girls and 20% of the boys. However at the age of 17 in girls and 18 in boys only 3.4% of the girls and only 0.6% of the boys are entirely free from comedones while 17% of the girls and 54% of the boys had clinical acne.

He found a striking correlation in the age of occurrence of acne and the age of appearance of axillary and pubic hair and the onset of menses.

Comparable data regarding incidence and age distribution were reported in a survey similar in design to that of Bloch including 2052 boys and girls between the ages of 5 and 22 in the Chicago area (7). There was no signific-

ant sexual difference in its incidence. Cohen (8) examining a group of 500 women between the ages of 17 and 40 found the maximum incidence between age 19 and 21. Despite the high degree of association between acne and adolescence it is seen also later in life and has been reported infrequently in infants (9).

An extensive review of the literature covering the etiology of acne vulgaris was made by Cohen (10) in 1941. He touched on such factors as diet, vitamin deficiency, defects of carbohydrate, fat or water metobolism, infection, allergy, thyroid or pituitary dysfunction, familial predisposition, psychoneurotic reaction, fatigue, iodides, bromides, and age-endocrine relations, all of which have been put forward at some time or another as important influences if not the cause of acne vulgaris. But the bulk of the review is devoted to summarizing the evidence regarding the influence of endocrine glands. He comments that "the strongest arguments in favor of an endocrine aetiology are the regularity of its appearance at puberty and its variation with the menses". He also points out that "acne does occur with abnormal frequency in conjunction with certain disorders of the endocrine system, such as tumours of the suprarenal cortex and arrhenoblastomata; also during the administration of androgens, such as testosterone propionate."

Investigation of the latter aspect was the substance of Hamilton's report (11) on the effects of the admin-

istration of androgens to eunuchoids and pre-pubertal boys. Acne, not known to occur in eunuchoids and castrates, developed in 7 of 9 of these while receiving testosterone. The acne varied in proportion to the amount of androgen given, cleared when therapy stopped, only to recur when it was resumed. Of 31 boys with cryptorchidism between 2 and 23 years of age, 22 developed acne while receiving testosterone. A high degree of correlation was noted between clinical improvement, i.e. sexual maturation, increase in urinary androgen, and acne. There was individual variation in the amount of testosterone, the level of urinary androgen, and the duration of treatment necessary to invoke acne. There was no apparent correlation between FSH levels and acne. Increased sebaceous secretion was observed during treatment.

In summary Hamilton stated that "susceptible individuals do not develop acne unless adequate gonodal hormone substance is present. Presumably factors of an infectious congenital or dietary nature exist prior to administration of the hormone but are in themselves ineffective in the absence of at least a certain degree of body stimulation by androgens (or possibly by other agents stimulating the pilosebaceous structures)".

Corroborative evidence for Hamilton's work is not lacking. Acne is a recognized complication of androgen therapy in the female, occurring in 8% of one series of 153 women treated for various gynecologic disorders (12).

That an excessive degree of body stimulation by androgens may be provided by an over active adrenal cortex is most strikingly demonstrated in those cases of the virilizing syndrome due to adrenal cortical tumor or hyperplasia. Soffer et al. (13) in describing the full blown clinical picture of the syndrome in the female list acne among the principal signs and symptoms. In the more recent case reports of the adrenogenital syndrome (virilization of adrenal origin) acne is noted more often than not and is reported in one case at the age of 15 months (14). All four of the cases reported by Venning et al. (15) showed acne as did all eight of Wilkins' series (16).

Despite the frequent occurrence of acne in the presence of adrenal virilism it is not an inevitable accompaniment of this condition. It is even less consistently reported in the type of adrenal hyperfunction manifested in Cushing's syndrome.

Soon after the therapeutic use of ACTH and cortisone became general reports began to appear in which the occurrence or aggravation of acne was observed in some patients during treatment with ACTH (17) (18) (19). Acne following cortisone therapy was not seen consistently or frequently and only following prolonged administration, viz., 187 days (19).

The apparent relationship between acne and androgenic hormones has been studied histologically in experimental

animals and less frequently in humans. The antagonistic action of estrogens in this respect has been clearly shown. In the rat estrogen administration is followed by thinning of all layers of the skin and a striking diminution in the size and number of sebaceous glands to the extent that many are almost obliterated. When testosterone and estrogen are administered together none of these changes appear and the skin remains almost identical with that of untreated controls (20). When testosterone alone is given there is an increase in the size and activity but not the number of sebaceous glands (21). Similar changes have been demonstrated in the rabbit (22).

In the androgen treated hamster Hamilton and Montagna (23) reported massive enlargement of the sebaceous glands with an increase in the number and size of cells, an increase in the sebum in the lumina of the acini, ducts, peripheral portions of hair follicles and on the surface of the skin. Thus the functional corrollary of the morphological changes in the sebaceous glands is made clear. It has been shown elsewhere (24) that the ratio of sebaceous gland secretion to sebaceous gland area in a given area of the skin is constant. In other words the amount of sebum produced depends on the number and size of sebaceous gland cells.

The comparative effect of testosterone, progesterone, cortisone and ACTH on the skin of the rat has been studied quantitatively by Haskin et al. (25). Hyperplasia

of the sebaceous glands was most pronounced in the animals receiving testosterone (1 mg. daily for 30 days). Progesterone (10 mg. daily for 15 days) produced comparable changes, while ACTH treatment showed only slight hyperplasia, and cortisone none.

In humans, Rony and Zakon (26) have demonstrated in punch biopsies of the pubic skin a thickening of the epidermis and a decided increase in the number and size of sebaceous glands following testosterone therapy. Their subjects were 6 boys aged 8 to 9, two with unilateral cryptorchidism and four with moderate obesity. The same investigators later showed similar changes in the scalp of two balding men (27). Estrogen reversed the changes in the scalp.

The evidence reviewed so far indicates that an increase in androgens thickens the epidermis and stimulates the sebaceous glands to excrete an increased amount of sebum. It is not difficult to see how comedo formation could result from these two changes or even from one or other of them alone. This of course does not exclude other factors which may be operating e.g. alteration in the chemical and physical nature of the sebum due to metabolic disturbances with resulting variation in viscosity.

The antagonistic action of estrogen and androgen manifested in the sebaceous gland has been utilized for many years in the treatment of acne. Theoretically

the results should be clear cut and uniformly beneficial. However the difficulties of assessing therapy in acne are the difficulties met with in all diseases characterized by remissions and exacerbations. This is well illustrated in a report by Lynch (28) who used estrogen ointment on 49 university girls with acne of whom 72% improved. In a control group treated with the ointment base alone 64% improved. Similar experiences have been reported using other therapeutic agents. Of a group of 35 patients receiving 100,000 I.U. of vitamin A daily, 20 showed improvement while of 8 given placebos 4 improved and 4 did not (29).

Some reports make the point that estrogen treatment is more effective in the male. However the majority make no distinction. Most reports are favorable in tone, some frankly enthusiastic. On the other hand a well controlled series of young men treated with diethylstilbestrol showed no improvement over the control group receiving routine therapy. Estrogen therapy in this case was judged a failure (30).

The question arises as to whether acne is consistently accompanied by elevated androgen levels or, alternatively, by normal androgen levels which are partly unopposed in their action on the skin by a deficiency of estrogen.

The earlier investigations of the endocrine status of acne patients were confined to estrogen assay. Rosenthal and Kurzrok (31) using bioassay methods found 83% of repeated

determinations on the urine of three normal women to be positive for estrogen whereas only 21% of determinations on the urine of 34 girls and women with acne were positive. In a fixed amount of blood withdrawn during the last ten days of the menstrual cycle Rosenthal and Neustaedter (32) found at least one mouse unit of estrogen in 75% of normal women. In 29 women with acne this level was attained in only 7%. The same method was used by McCarthy and Hunter (33) who found only one of 11 controls deficient in estrogen, whereas 41 of 60 women with acne showed a deficiency by this standard.

Bioassay of a simple choloroform extract of mid-cycle urine was found to yield an average of 7.7 rat units per litre in 20 control women by Wile, Barney and Bradbury (34) while in 12 women with severe acne the yield averaged 4.1 rat unites per litre. A subsequent report by Wile, Snow and Bradbury (35) included assay of androgens by the capon comb growth method and studied men as well as women. The 24 hour excretion of androgens by acne patients in both sexes was slightly increased over control values while, again in both sexes, the estrogen excretion was less in the acne patients than in controls. Thus the ratio of androgen to estrogen excretion appeared as a distinctive index. Cornbleet and Barnes (36) using a comb growth test found that patients with acne excrete quantities of androgen which lie at the lower part of the normal range. No estrogen assay was attempted. It is interesting to note that they reported

some success with testosterone treatment. Lawrence and Werthessen (37) also found that androgen excretion (measured colorimetrically) was slightly but not significantly less in 8 women with acne as compared with 8 normal women. The estrogen excretion (measured by bioassy) however was significantly less in the acne group. They concluded that in the female a disturbance of the androgen: estrogen ratio was a significant etiological factor in acne.

More recently two series have been reported in which 17-ketosteroid excretion in patients with acne has been shown to be within normal limits (38) or without any significant increase (39). Yet another series (40) is reported in which 17-ketosteroid values were abnormally high.

It would appear then that the endocrine disorder in acne vulgares may be a relative excess of androgenic hormone. Estrogen deficiency alone cannot be the cause as Hamilton (11) pointed out, in that children and eunuchs with low estrogen levels do not have acne. At the same time he found in his series as wide a variation in the androgen: estrogen ratio among those who developed acne as among those who did not. There appears to be at least one additional factor involved which may come under the heading of familial or hereditary predisposition. Bloch (6) refers to the possibility that ".....the follicular apparatus of the skin (the 'receptor mechanism') is individually

different in its sensibility to.....(the sexual hormone)". And Sulzberger (41) gives a place in etiology to the "susceptibility or readiness of the organ of response".

The Problem

If an excess of androgen underlies the occurrence of acne in both sexes then it would be expected that the adrenal cortex which supplies most of the androgen in the male and practically all in the female (as judged by 17-ketosteroid excretion) would play a major role in the pathogenesis of the disease. The fact that both androgens and estrogens are derived from the adrenal cortex suggests that some abnormal pattern of activity may exist in patients with persistent acne. It is obvious that such is the case in the acne associated with adrenal cortical hyperplasia and tumor and with the administration of ATCH.

Providing that the generally accepted indices of adrenal cortical activity are sufficiently sensitive it should be possible to demonstrate in patients with persistent acne a secretion pattern favoring an excess of circulating androgen. The likelihood of discovering such a pattern would probably be increased by stimulation of the adrenal cortex by ACTH.

Method

a. General

In order to examine this hypothesis it was decided to study a small group of patients, men and women, who suffered from persistent acne of at least three years' duration and to compare the results with those of a control group of similar age range.

The indices of greatest significance were considered to be urinary 17-ketosteroid and cortin levels. In addition, in view of the apparent antagonism of androgen and estrogen in acne it was decided to include data on estrogen excretion providing a suitable method for its determination could be developed.

b. Clinical Methods

Patients were drawn in part by referral from dermatological and other medical practices and in part from members of the hospital staff. Cases of short-lived adolescent acne were avoided by excluding those with less than a three year history and concentrating on the older age group. All but one case (age 18) were over 21 and the majority had a severe degree of acne.

Controls included one patient under investigation for migraine type headache (K.M.) who was otherwise in good health. The remainder were either internes or hospital employees. An attempt was made to match the patients with

controls of comparable age.

The plan of investigation involved 24-hour urine collections for three consecutive days (8 a.m. - 8 a.m.) with the administration of ACTH intravenously on the second day. Subjects were admitted to hospital usually on the evening of the first day but occasionally on the morning of the second day. Some were discharged on the second day, following the completion of the intravenous infusion, while others remained varying periods up to the end of the third day of urine collection.

The routine investigation for all consisted of history, physical examination, white blood cell and differential count, hemoglobin, hematocrit, sedimentation rate determinations and urinalysis. Eosinophil counts (method of Randolph, T.G., J. Lab. & Clin. Med., 34: 1696, 1949) were done before and near the conclusion of the six to seven hour intravenous infusion of 25 mg. of ACTH in 500 ml. of physiological saline. All received ACTH of the same lot except H.M., V.W. and K.M.

c. Chemical Methods

Urine was collected in specially cleaned bottles without preservative. One hundred ml. aliquots were taken for 17-ketosteroid, cortin, and estrogen assay. When assays could not be done immediately specimens were refrigerated for short periods (up to 24 hours) or frozen for longer per-

iods. Creatinine determinations were done on all 24 hour specimens.

The 17-ketoseroids were determined as described by Holtorff and Koch (42). Cortin assays (determination of formaldehydogenic steroids) were performed by the method of Daughaday, Jaffe and Williams (43) as modified by Tompsett and Oastler (44).

Estrogen assay is described in appendix A. To indicate the lack of specificity of the technique the results are labelled "fluorogenic phenols".

Results

The clinical particulars of the patients and controls are given in Table I. The results of routine blood and urine examination were within normal limits in all cases. Severity of acne was graded on examination as mild, moderate or severe.

The results of the steroid assays are shown in Table II (where results of the determinations done on the first, second and third days of the investigation are labelled D1, D2 and D3 respectively) and in Figures 1. and 2. As the method used for estrogen determination proved to be highly non-specific (see Appendix A) the results (Fluorogenic Phenols) are included with the reservation that they are of questionable significance.

It will be seen that there is considerable variation in individual patterns of excretion within each group. No obvious and consistent difference characterizes the acne group unless it be an overall hyperactivity in all aspects measured. Despite the degree of individual variation the difficulty of obtaining additional acne patients and more particularly controls provoked the analysis of the existing data by small sample techniques (45) to ascertain if significant differences had already been established.

In the statistical analysis the males with and without acne were treated quite separately from the females

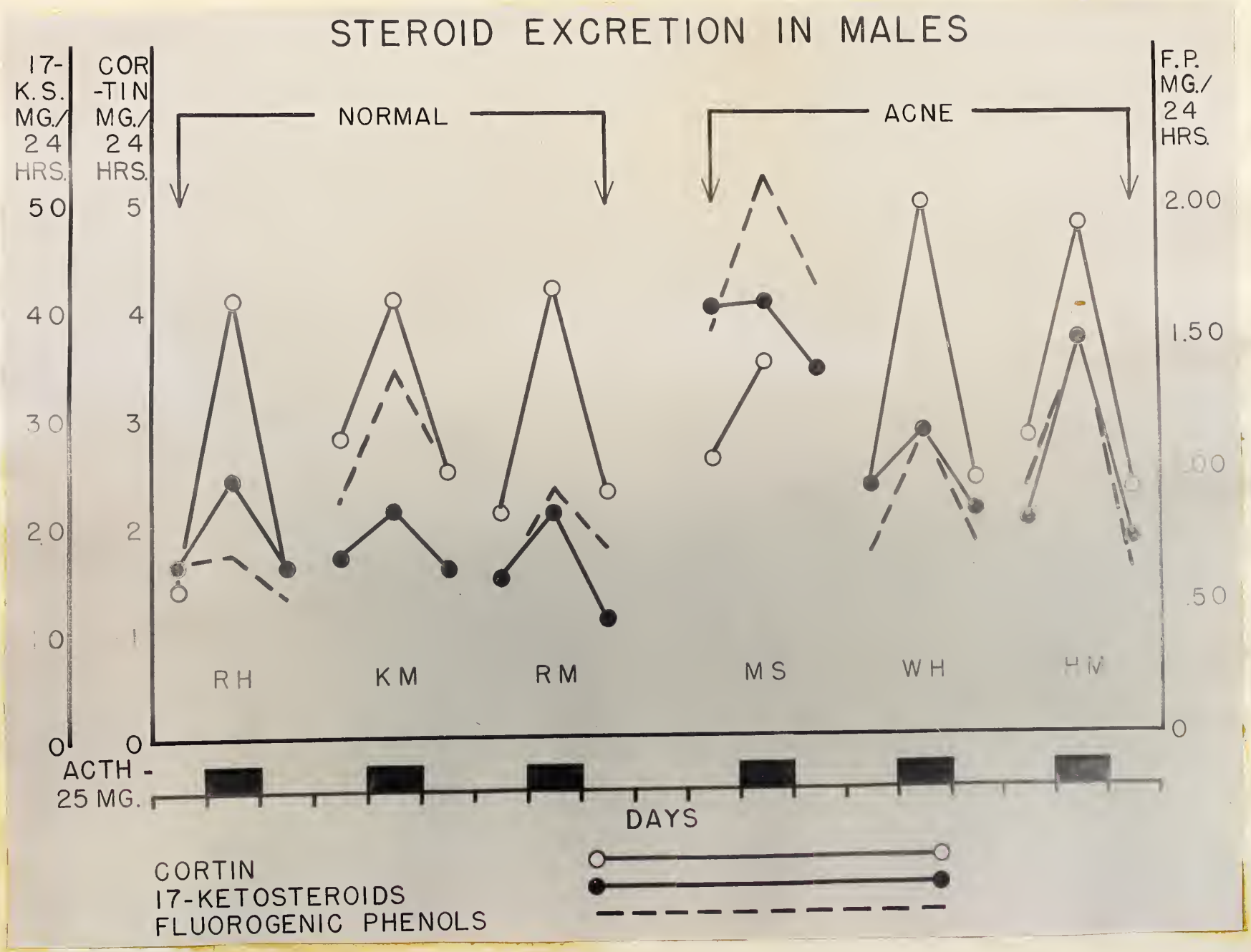

STEROID EXCRETION IN MALES
17-K.S. MG./24 HRS.
COR-TIN MG./24 HRS.
F.P. MG./24 HRS.
NORMAL
ACNE
50
40
30
20
10
0
5
4
3
2
1
0
2.00
1.50
1.00
.50
0
RH
KM
RM
MS
WH
HM
ACTH - 25 MG.
DAYS
CORTIN
17-KETOSTEROIDS
FLUOROGENIC PHENOLS

Figure 1.

TABLE I. Clinical Particulars

University Hospital Number	Patient	Sex	Age	Duration of acne	Severity of acne	Menstrual irregularity
119812	RH	M	23			
118985	KM	M	27			
120475	RM	M	35			
119626	MS	M	21	7 yrs.	v. severe	
120866	WH	M	26	12 yrs.	severe	
119407	HM	M	32	18 yrs.	severe	
120807	CB	F	18			none
117807	GL	F	20			none
120018	EM	F	28			none
119255	VW	F	18	3 yrs.	moderate	4 yrs. secondary amenorrhea
97364	MC	F	22	10 yrs.	severe	4 to 10 week intervals
119644	PF	F	22	10 yrs.	mild	4 to 12 month intervals

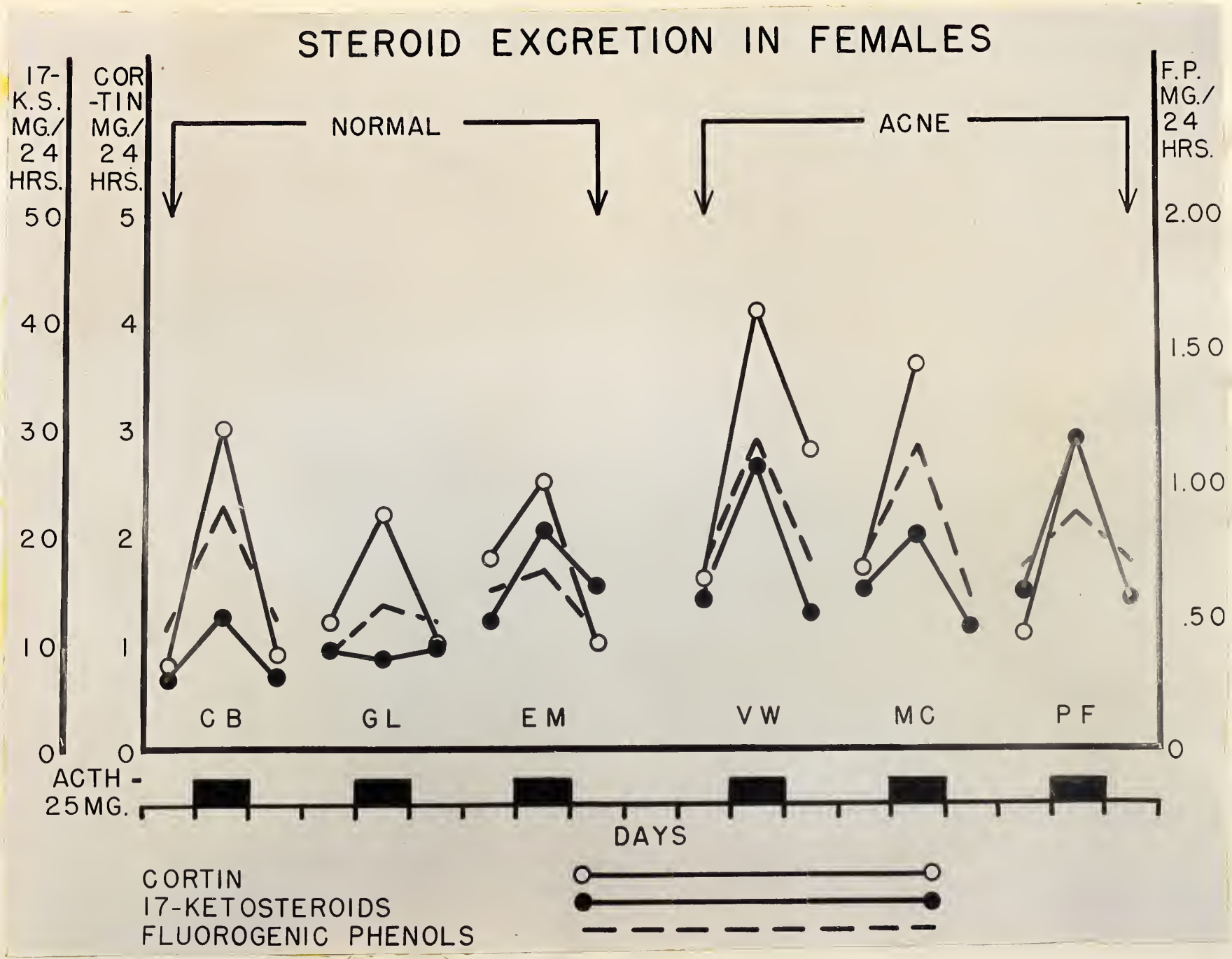

STEROID EXCRETION IN FEMALES
17-K.S. MG./24 HRS.
CORTIN MG./24 HRS.
F.P. MG./24 HRS.
NORMAL
ACNE
50
40
30
20
10
0
5
4
3
2
1
0
2.00
1.50
1.00
.50
0
CB
GL
EM
VW
MC
PF
ACTH - 25MG.
DAYS
CORTIN
17-KETOSTEROIDS
FLUOROGENIC PHENOLS

Figure 2.

TABLE II. Steroid assays.

Patient	D1			D2			D3			Eosinophile response
	K.S. mg.	Cortin mg.	F.P. mg.	K.S. mg.	Cortin mg.	F.P. mg.	K.S. mg.	Cortin mg.	F.P. mg.	
RH	16.2	1.4	.661	24.2	4.1	.693	16.0	1.6	.528	100%
KM	16.9	2.8	.880	21.2	4.1	1.382	15.9	2.5	.996	20%
RM	15.1	2.1	.568	21.2	4.2	.935	10.9	2.3	.710	100%
MS	40.6	2.6	1.520	41.5	3.5	2.102	34.4	lost	1.692	41%
WH	23.4	2.3	.678	28.6	5.0	1.142	21.2	2.4	.723	85%
HM	20.1	2.8	.924	37.2	4.8	1.475	18.3	2.3	.620	75%
CB	6.7	0.8	.454	12.5	3.0	.912	6.9	0.9	.490	100%
GL	9.4	1.2	.357	8.5	2.2	.539	9.7	1.0	.476	79%
EM	12.1	1.8	.594	20.6	2.5	.669	15.3	1.0	.437	88%
VW	14.1	1.6	.663	26.4	4.1	1.156	12.8	2.8	.697	40%
MC	15.1	1.7	.720	20.1	3.6	1.127	11.6	lost	.565	70%
PF	14.8	1.1	.686	29.0	2.9	.884	14.3	1.4	.696	54%

K.S. = 17-ketosteroids. F.P. = Fluorogenic Phenols

with and without acne. Androgen: estrogen ratios were not studied because of the unreliable estrogen data obtained. The differences in cortin values although following the same trends as the 17-ketosteroids were seen to be less pronounced. Comparison of male cortin values on D2 showed no significant difference while the same comparison in females revealed a barely suggestive difference (P between 0.1 and 0.05).

With regard to 17-ketosteroids in males there was no significant difference in the D1 values (attributed to the large variation introduced by M.S.) nor in the increase of D2 over D1 but in the D2 values themselves the difference between acne and normal groups was highly significant.

In females the 17-ketosteroid values for D1 showed a highly significant difference between the acne and normal groups. The increase to D2 was not significantly different and the D2 values showed only a suggestive difference (P between 0.1 and 0.05).

Thus it was concluded that within the limits of this investigation a significant difference between acne and normal groups may lie in an increased 17-ketosteroid excretion in the former.

Discussion

The small number of subjects studied in this investigation reduces it to a purely exploratory level. However an impression is gained that acne patients start with a slightly higher level of adrenal cortical activity than normals and that under the influence of ACTH both groups show an increase in activity proportional to their pre-treatment levels. Thus in comparing the increase in 17-ketosteroid excretion from D1 to D2 in the acne patients and in normals (combining males and females in each case) the percentage increase is 41.9 and 41.6% respectively. This demonstrates that ACTH administration adds little to the information gained by determinations on a random 24-hour specimen. The D3 values show merely varying degrees of return to pre-treatment levels.

Proceeding on the basis of these conclusions a supplementary series has been started and although still incomplete the same tendency for increased 17-ketosteroid excretion in acne is seen (see Appendix B).

Thus the answer to the problem as to whether acne is consistently accompanied by an abnormal pattern of adrenal activity is not definitely answered. This requires extension of the supplementary series to the stage where statistically significant differences can be shown between the two groups. In view of the antagonism between androgen and estrogen in the skin final proof may also require the

use of improved estrogen assay methods. This is suggested by the occurrence of normal 17-ketosteroid levels in occasional acne patients.

Another factor to be taken into consideration is one of the time relations in assessing adrenal activity by means of 17-ketosteroid excretion. All results in this study are based on 24-hour urine specimens. But Pincus (46) has shown that increased excretion can be detected in periods as short as two hours following the initiation of psychic stress. Thus in the present study if unusual peaks of excretion occurred over short periods they would have been largely obscured in the 24-hour volume. Further discussion of the implications of this enters the realm of conjecture. The studies of Robin and Kepecs (47) and Lorenz, Graham and Wolf (48) indicate that intensive emotional activity is accompanied by increased sebum excretion. The possibility that adrenal androgens produced under psychic stress have an immediate effect on the sebaceous glands is purely hypothetical. But Lorenz et al. postulate that it is the alternation of short periods of sebaceous hyper-excretion in anger and longer periods of hypo-excretion in remorse that leads to plugging of the hair follicle and comedo formation.

Also conjectural is the influence of progesterone in women with acne. Haskin et al. (25) showed its testosterone-like effect on the rat sebaceous gland.

Selye (49) attributes to it some slight testoid activity while Aron-Brunetiere (50) includes progesterone with androgen in discussing the androgen: estrogen ratio. It may have some influence in the pre-menstrual exacerbation of acne reported by so many women.

It is obvious that psychogenic mechanisms mediated through the adrenal cortex are not the whole answer to the problem of acne vulgaris for this does not explain its frequent occurrence in cases of adrenal hyperplasia.

One sees acne almost physiologically in adolescence and again as an inconstant symptom of conditions in which excessive adrenal cortical activity is the fundamental pathological process; as well as in other diseases associated with excessive androgen secretion. There remains a group of patients in whom acne persists into the third or fourth decade and who show no other signs of increased androgenic stimulation, and a transitional group including females with acne and various menstrual irregularities, amenorrhea, or relative sterility.

APPENDIX A

ESTROGEN DETERMINATION

Introduction

The chief requirement for the method besides reasonable accuracy and specificity was a high degree of sensitivity in order that the small amount of estrogen in male and non-pregnancy female urines could be detected. This amount is thought to be of the order of 50 mcg./24 hours as compared with amounts as high as 40 mg./24 hours in late pregnancy (55). A further consideration was a degree of simplicity sufficient to allow the determinations to be done in the hospital laboratory under the same conditions and in the same time required for the other steroid assays.

The usual bioassay methods, although sensitive, had the disadvantage of involving large numbers of animals over several days for each determination. In addition serios errors may be introduced when separation of the three known estrogens is incomplete due to their differing biological potencies. A chemical method circumventing these difficulties but with comparable accuracy specificity and sensitivity seemed preferable.

With these requirements in mind various extraction and separation procedures were studied and an attempt

was made to evaluate the existing micro-methods for final assay. Thus the problem devolved into two parts: the hydrolysis, extraction and separation of the estrogens, which is common to bioassay and chemical assay; and the measurement of their concentration in a purified extract.

It soon became apparent that the older methods of hydrolysis, extraction and separation were often based on inadequately verified data. Moreover the material studied was invariably pregnancy urine with its very high content of estrogen. Fortunately there have appeared in the past few years at least two comprehensive reviews of existing methods. Friedgood and Garst (56) used ultra-violet spectrophotometry to verify the efficiency of each step in extraction and separation and proposed a new method incorporating their results. The limitation of this report lies in the fact that their data were based only on samples of pure estrogen and the method was not applied to urine. Engel (57) similarly investigated numer-ous solvent partition procedures. Partition co-efficients for each of the three estrogens in various solvent pairs were derived and applied in drawing up a recommended extraction and separation procedure. The authenticity of the method was proved by the results of counter-current distribution studies and fluorometric analysis. It is noteworthy that although Engel's report dealt largely with pregnancy urine the partition co-efficients concerned are

particularly applicable to dilute solutions and in dilute solutions they are not affected by the presence of other solutes. Engel had some success in the assay of non-pregnancy urines.

Liquid chromatography was investigated to some extent. It had the merit of combining some elements of extraction, purification and separation. However the time available was judged too short to institute an entirely new technique. This consideration was even more applicable to paper chromatography.

Thus it appeared that Engel's method was the most reliable solvent partition procedure available and the details of the initial hydrolysis, extraction and separation were adopted. Friedgood and Garst gave a complete description of the separation of estrone, estradiol and estriol which was adopted.

Of the micro-methods for final analysis ultraviolet spectrophotometry was eliminated because of its inadequate sensitivity, Friedgood and Garst having stated that 12 to 15 mcg./ml. was the lower limit of the concentration that could be measured. Polarography was not investigated although this has been used in an attempt to determine the progesterone content of plasma. Fluorometry remained as the most promising possibility.

Several methods of developing and measuring the fluorescence of estrogens were available. One of the early

methods was described by Jailer (58) who heated estrogens with sulfuric acid and developed a procedure for the analysis of urinary extracts. The sulfuric acid method, as modified by Bates and Cohen (59) (60) and Engel, Slaunwhite, Carter and Nathanson (61) has been widely used. Garst, Nyc, Maron and Friedgood (62) developed a method depending on condensation of estrogens with phthalic anhydride. Finkelstein, Hestrin and Koch (63) (64) (65) described a procedure in which estrogens were heated with phosphoric acid. All these methods were highly sensitive and reasonably accurate.

An experimental investigation and comparison of the above methods (excepting that of Garst et al.) was carried out by Braunsberg (66). The method of Bates and Cohen proved to be most sensitive but it was concluded that the method of Finkelstein et al. might be less influenced by any impurities present because of the use of the less reactive phosphoric acid. Accordingly this method was selected for trial.

The composite method now included with slight modifications the initial extraction of combined estrogens according to Engel, the separation of estrone, estradiol and estriol as described by Friedgood and Garst and the fluorescence development and measurement of Finkelstein.

In the initial trials of the entire procedure the hydrolysis, extraction and separation of the combined

estrogens from the neutral steroids consumed some three to four hours' time. Continuation of the procedure to separate the three estrogens one from the other would extend the work into a second day. In view of this it was decided to dispense with the separation and individual determination of the three estrogens on the assumption that the measurement of the combined estrogens would suffice to detect abnormalities in clinical cases. Accordingly the following procedure was adopted.

Method

The 24-hour urine was collected without preservative and the quantity measured. Of this 100 ml. was taken for analysis.

100 ml. urine + 7 ml. conc. HCl

↓

autoclave 20 min. at 15-20 lbs. pressure

cool under tap-water

↓

extract with 4 x 20 ml. ether

↓

wash with 2 x 7 ml. saturated $NaHCO_3$ and 4 ml. water

↓

evaporate to dryness at 60° C

dissolve residue in 75 ml. toluene

|

extract with 4 x 20 ml. 1N NaOH
and 2 x 4 ml. water

|

(toluene containing neutral steroids discarded)

|

acidify to pH 9 ± 0.5 with 6N H_2SO_4
using Hydrion paper

add 2 ml. $KHCO_3/K_2CO_3$ buffer solution

(20 vols. sat. $KHCO_3$: 1 vol. sat. K_2CO_3)

|

extract with 4 x 20 ml. ether

|

evaporate to dryness at 60° C

add 4 ml. ketone-free ethanol

|

place aliquots (usually 1/20) in test tubes
provided with tight-fitting glass stoppers

heat in oven at 110° C for 40 min.

|

add 8 ml. conc. H_3PO_4

stopper tubes

|

heat in boiling water both 30 min. in
the dark, shaking for first 2 min.

cool to room temp in water bath

transfer to cuvettes

read in fluorometer

Reagents and Special Equipment

Reagent quality chemicals were used unless otherwise indicated. Ethyl ether was redistilled after washing with ferrous sulfate and water. Ketone-free ethanol was prepared by adding 5 Gm./litre phenylenediamine hydrochloride and storing one week with periodic shaking. It was refluxed one hour and distilled discarding the first 50 ml. and the last 100 ml. then redistilled. No steps were taken to purify the toluene. Dow Corning stopcock grease was used. This was found to have no fluorescent activity after heating with phosphoric acid.

For hydrolysis a No. 4 National (Presto) Cooker-Canner was used. Special 150 x 16 mm. pyrex test tubes with ground glass stoppers were obtained to take the aliquots of the final estrogen extract in ethanol. After drying and adding acid the stoppers were tightly fitted to prevent ingress of moisture while in the water bath. The water bath was specially made to allow the rack holding the tubes to be rotated 90° either way from the vertical by an outside handle. A tight-fitting lid kept out light while steam escaped by way of a U-shaped vent. This

arrangement allowed the contents of the tubes to be mixed while being heated in the dark.

The Fluorometer

The instrument used was a model 12A Coleman Photofluorometer. This is a direct reading instrument designed with a single light path extending from the source, a mercury vapor lamp, through the primary (optical) filter to the cuvette well, then through the secondary (photocell) filter to the recording photocell circuit. Thus variations in the intensity of the light at the source influence the readings, a difficulty largely overcome by instruments with a balanced system comprising two photocells, a measuring and a balancing cell, in a bridge circuit.

As a consequence of this deficiency much depends on the preliminary adjustment of the instrument in relation to a reference solution of standard fluorescent activity. There is, however, a limit to the range of adjustment which can be made and this is often exceeded by the variation in intensity of the light source. It is therefore necessary to control the latter within certain limits by external means.

The voltage of the power supply is an important factor in controlling the light intensity. It was found necessary to introduce into the circuit besides the autotransformer supplied with the instrument, first a voltage

stabilizer and secondly a variable transformer, the latter because the stabilizer delivered a constant but too high voltage to the instrument. A second important factor was the individual variation of different mercury vapor lamps and their slow decline in intensity with use. It was unfortunate that in this investigation the lamp first used was considerably more powerful than any of the replacements later obtained. Thus the standardized conditions of operation selected initially could not again be reached once the original lamp had declined appreciably in intensity and in order to restore these original conditions it was necessary to increase the voltage of the power supplied to the instrument beyond its recommended limits. This resulted in frequent lamp failures often at critical moments which in turn were largely overcome by carefully timing the end of the period of stabilization (8 minutes) at increased voltage to co-incide with the time for reading (10 minutes after completion of heating in the dark). In this way the period of high voltage was kept to a minimum.

The reference solution originally selected was fluorescein sodium (sodium fluoresceinate) made by mixing 10 mg. fluorescein and 15 mg. sodium bicarbonate in a litre of distilled water. Several days were allowed for solution. The behavior of this reference solution (in a dilution containing 0.08 mcg./ml.) was studied intermittently over a

period of about one month. This was at a time when the power supply to the instrument had been stabilized at the recommended voltage but before it had been realized that the light intensity of the mercury vapor lamp decreased slightly with use. Thus although day to day variation in readings obtained with the reference solution might be attributed to uncontrollable minor variations in performance of the instrument the overall trend during this period revealed a decrease in activity which threatened in time to exceed the range of adjustment of the instrument. In view of the uncertainty as to whether this was due to fading of the lamp or of the reference solution it was decided to change to quinine sulfate, a reference solution which had been more widely used and whose stability could be relied upon.

A minor disadvantage in using quinine sulfate was that its absorption and fluorescent maxima were sufficiently different from those of phosphoric acid-treated estrogens as to require a different set of filters. (This had not applied to fluorescein.) The solution was made by dissolving 10 mg. quinine sulfate in a litre of N/10 sulfuric acid. This was kept in a refrigerator. Fresh dilutions were made daily by taking 0.5 ml. of the refrigerated solution and making up to 25 ml. in a volumetric flask with N/10 sulfuric acid. Eight ml. of this (0.2 mcg./ml.) were used as a reference. After a month

of use in which gradually decreasing readings were obtained it became apparent that the lamp was at fault. When maximum adjustment of the instrument failed to maintain the desired scale reading this was rectified as has been mentioned by increasing the voltage of the power supply. Quinine sulfate continued to be used.

Filters

Finkelstein used a primary filter transmitting at 436 mu. and a secondary filter transmitting at 530 mu. Studies of the absorption spectra of the phosphoric acid-treated estrogens by Braunsberg (66) indicated that 460 mu. was the optimal wave length for excitation but that 436 mu. was acceptable. This compromise is imposed by the nature of the light source, a mercury vapor lamp, one of whose principal lines must be chosen. Goldzieher et al. (67) measured the fluorescence spectra of acid-treated estrogens excited by the principal lines of the mercury arc and found that the 436 mu. line produced maximum fluorescence. Fluorescence peaks were at 500 mu. for estradiol and estrone, and at 525 mu. for estriol.

Thus the B2 (436 mu.) and PC2 (530 mu.+) filters provided with the photofluorometer are satisfactory. Quinine sulfate fluoresces at a much shorter wave length for which the B1 (365 mu.) and PC1 (420 mu.+) filters are suitable.

Experimental Conditions

1. Emulsions and water washes of the first ether extract.

Troublesome emulsions often occurred during the initial ether extraction. The two sodium bicarbonate and single water washes helped to break these but in some cases they persisted. It was thought that additional water washes might be of value in spite of the danger of losing some of the hydrophilic estriol. Accordingly duplicate assays were done on two different days using aliquots of the same 24-hour urine. Each day one specimen received one water wash and the other three. Results are shown in Table 1.

TABLE 1. Effect of water washes on first extract.

day	number of washes	scale reading
first	1	82
	3	77
second	1	81
	3	82

The scale reading recorded above was obtained with one tenth of the final estrogen extract. One tenth of the extra washes of the second day was determined fluorimetically and gave a scale reading of 28. There was no way of telling whether any of this fluorescence represented

estrogen.

Subsequently it was found that with careful handling emulsions could be broken without additional water washes.

2. Drying time at 110° C.

Finkelstein gave a drying time of one hour. In order to determine if this could be shortened samples of pure estrogen were subjected to the final steps of the analysis with varying drying periods. Results are shown in Table 2. in which 1 mcg. of estrone in 1 ml. ethanol per tube was used.

TABLE 2. Effect of varying drying time.

tube no.	drying time	scale reading
1	20 min.	30
2	20 min.	28
3	30 min.	35
4	30 min.	40
5	40 min.	38
6	40 min.	29

The variation between duplicate tubes dried for the same time was so great that the experiment was repeated using 2 mcg. estriol in 0.5 ml. ethanol per tube with the res-

ults shown in Table 3.

TABLE 3. Effect of varying drying time.

tube no.	drying time	scale reading
1	30 min.	25
2	30 min.	35
3	45 min.	28
4	45 min.	33
5	60 min.	26
6	60 min.	31

Again there was great variation between duplicate tubes. All had been subject to the same conditions apart from drying time except that the first tube of each pair had received acid from the first filling of the burette and the second tube of each pair from the second filling of the burette. Thus the major difference in results was attributed to differences in the acid added. Within each series receiving acid from the same buretteful the variation with time of drying was insignificant, viz., 25, 28, 26 and 35, 33, 31 for 30, 45 and 60 minutes respectively. An arbitrary drying time of 40 minutes was therefore selected.

3. Effect of acid on fluorescence.

Concentrated (85%) phosphoric acid alone has some

fluorescent activity which varies considerably from bottle to bottle. This difficulty is largely overcome by carrying a blank tube of acid through the final heating procedure. The photofluorometer used allows the scale reading to be set to zero with the blank in place. The difference revealed in the previous experiment between the two burettefuls of acid from the same bottle can be explained on the basis of the hygroscopic nature of the acid. The 25 ml. burette used for measuring the 8 ml. of acid into each tube was customarily left empty after use and refilled from the bottle when subsequently required. In this way due to the pronounced viscosity of the acid a fine film lining the inside of the burette would be exposed to air and mixed with the next filling of acid. This contaminated acid would be distributed in the first three tubes filled. The next three tubes filled would have the benefit of the previous rinsing and suffer less from contamination with water. This difference is shown in Table 4. in which tubes containing 2 mcg. estriol in 0.5 ml. ethanol were used.

TABLE 4. Effect of acid exposed to air.

tube no.	burette	scale reading
1	first filling	48
2	first filling	47
3	second filling	58
4	second filling	53

To obviate this effect it became standard practice to rinse the burette twice with 5 ml. of acid before use.

4. Effect of voltage stabilizer on fluorescence.

The introduction of the voltage stabilizer had one disadvantage. Its output was 1.04 amperes at 127 volts whereas the photofluorometer required 115 volts and 150 watts. Table 5 shows the loss of sensitivity involved in using the stabilizer when tested against aliquots of a urinary extract.

TABLE 5. Effect of stabilizer on fluorescence.

aliquot in ml.	scale reading	
	without stabilizer	with stabilizer
0.05	17	6
0.10	32	11
0.20	62	21
0.30	87	30
0.40	100+	39

Replicates

In order to test the accuracy (reproducibility) of the method, replicate determinations were done on a single 24-hour specimen of mid-cycle urine aliquots of

which were preserved by freezing until required. One tenth of the final extract was taken for fluorometry. Results are shown in Table 6.

TABLE 6. Replicate determinations.

date	100 ml. aliquot	scale reading
Dec. 16	1	63
	2	50
Dec. 18	3	55
	4	53
Dec. 23	5	54
	6	55
Jan. 8	7	82
	8	95
Jan. 13	9	82
	10	77
Jan. 15	11	81
	12	82

It will be seen that the December determinations differ substantially from the January determinations. The reason for this could not be found. However within each group the correspondence is good except for one aberrant result.

During the course of subsequent recovery experiments repeated determinations were done on two other 24-hour urines with results shown in Table 7. In these cases one twentieth of the final estrogen extract was taken for fluorometry.

TABLE 7. Further replicate determinations.

100 ml. aliquot	scale reading	
	urine II	urine III
1	33	53
2	27	51
3	34	52
4	34	47
5	-	47

Calibration

In preliminary trials it became evident that the fluorescent activity of very dilute solutions of pure crystalline estrogen in ethanol deteriorated rapidly with time. Therefore stock solutions containing 50 mcg./ml. were made in the expectation that these would remain stable for some months.

Satisfactory straight line fluorescence-concentration relations were demonstrated for estradiol, estrone, and estriol using from four to seven tubes in repeated calibrations. The only irregularity noticed was a slight tendency to a staircase effect in estradiol (one step) and estriol (two steps). This was not seen in estrone and therefore it was concluded that it was related to the number of hydroxyl groups in the molecule concerned. The

magnitude of this irregularity was not considered excessive.

It had been supposed that a reproducible calibration curve could be made for each of the estrogens from which the concentration of unknown solutions could be deduced. However irregular day to day variation was beyond acceptable limits. A search was made for factors which might account for this. Compensation for some of the instrumental errors in the fluorometer have been previously described. Errors inherent in the dilution of the stock solutions and the subsequent handling in pipettes were studied. From the stock solution of estriol containing 50 mcg./ml. two dilute solutions were prepared using independent equipment and aliquots containing 1, 2 and 3 mcg. respectively from each diluted solution were placed in different sets of tubes and determined fluorometrically. The results shown in Table 8 indicate that manipulation of stock solutions is not a source of error.

TABLE 8. Comparison of two dilutions of stock solution.

estriol mcg.	scale reading	
	dilution a.	dilution b.
1	20	21
2	39	37
3	52	57

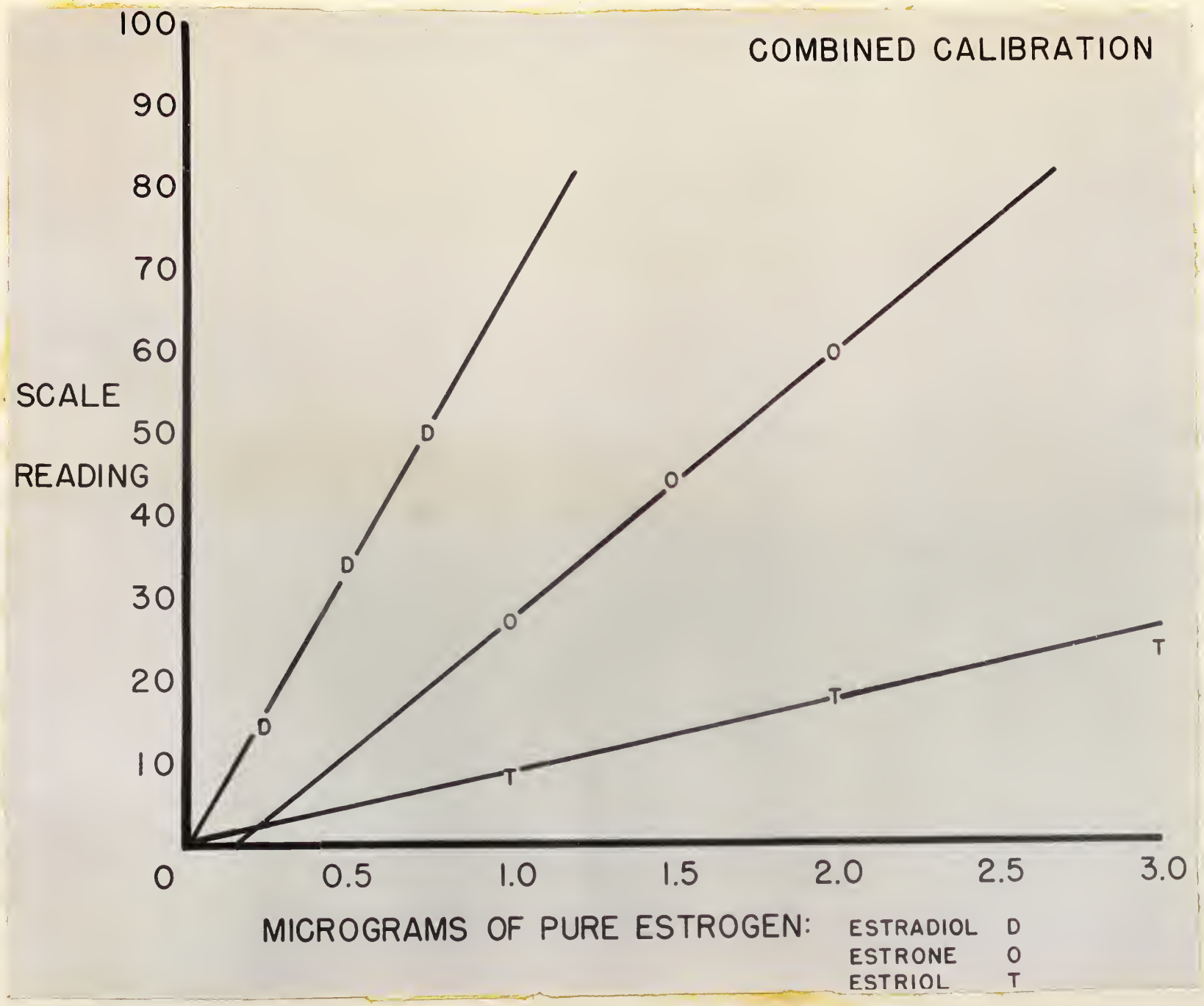

COMBINED CALIBRATION
SCALE READING
100
90
80
70
60
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
3.0
D
O
T
MICROGRAMS OF PURE ESTROGEN:
ESTRADIOL D
ESTRONE O
ESTRIOL T

Figure 3.

In order to investigate the possibility that some factor was affecting one estrogen and not the others or that it was affecting them unequally a combined calibration was done with all three estrogens simultaneously and repeated after intervals of 22 and 10 days. Since the maximum number of tubes that could be handled at one time was nine only three concentrations of each estrogen could be used. The results of the second calibration are shown in Figure 3. Comparison of this with the first and third graphs showed a regularly decreasing slope in all three curves with time. Thus the conclusion that even 50 mcg./ml. was an inadequate concentration for stock solutions could not be avoided. This deterioration however accounted only for a hitherto unnoticed downward trend in activity. An overall irregularity remained.

Figure 3 illustrates a characteristic of the phosphoric acid method of developing fluorescence which is of some theoretical value when dealing with a combined estrogen extract. The close similarity of the fluorescence spectra of all three estrogens has precluded their differentiation in a mixture by measurement of fluorescence at different wave lengths. The next most desirable situation would be that all three estrogens should fluoresce to the same extent, molecule for molecule, in which case the total amount of estrogen could be deduced. This does not occur in any method so far described. Failing this it

would seem most useful to have some correlation between biological and fluorescent activity. In this respect the phosphoric acid method is superior to any other. Infact, it is the only method in which the order of fluorescent activity is estradiol, estrone, estriol.

At about this stage in the investigation of the method, and with realization of some of its shortcomings, it was decided to proceed with recovery determinations and to continue improvements at the same time.

Recovery Experiments

After several preliminary trials the following procedure was adopted. A measured amount of the hormone in ethanol solution was added to the hydrolysate immediately before the first ether extraction. Since only one twentieth of the final estrogen extract was to be taken for assay, one twentieth of the amount of hormone added was also placed in a boiling tube to act as a standard. The extremely varied results are shown in Table 9. These cast serious doubt on the reliability of the method.

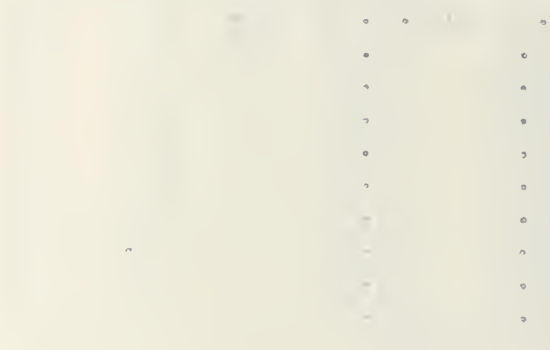
TABLE 9. Recovery experiments.

estrogen	amount added	amount assayed	scale reading: estrogen standard	scale reading: urine alone	scale reading: urine + estrogen	recovery %
estradiol	9.6 mcg.	0.48 mcg.	32	33	65	100
"	10.3	0.52	41	27	62	85
estrone	10.0	0.50	8	58	68	125
"	20.0	1.00	28	51	67	57
"	20.0	1.00	16	51	63	75
"	20.0	1.00	27	47	60	48
"	20.0	1.00	36	47	63	44
estriol	20.0	1.00	8.5	50	57	82
"	20.0	1.00	12	47	50	25
"	20.0	1.00	10	47	54	70

Experimental Conditions (continued)

5. Extent of straight line fluorescence-concentration relations.

Although satisfactory straight lines had been produced in individual calibrations with pure estrogen, observations with aliquots of urinary extracts raised the question as to whether a certain amount of self-absorption of fluorescence ("quenching") was occurring in these impure solutions. Accordingly, assays of increasing amounts of the same urinary extract were carried out. The lines produced in three different experiments with different extracts were satisfactorily straight except at the higher concentrations where a slight falling off occurred. An amount equal to one twentieth of the extracts fell within the straight portion of the lines and this amount was thereafter the maximum aliquot taken for assay.



6. Quenching of fluorescence.

It remained to investigate the possibility of quenching occurring in a mixture of urinary extract and added estrogen. The first experiments compared the fluorescence of estrone in 4 concentrations in the presence of a fixed amount of urinary extract and alone. Results are shown in Table 10.

TABLE 10. Quenching of estrone.

	scale reading			
	0.25	0.50	0.75	1.0 mcg.
estrone alone	10	25	41	53
with extract I	5	8	13	15
estrone alone	6	14	21	33
with extract II	1	6	8	7

This extreme quenching of the fluorescence of estrone by two different urinary extracts was not seen when estradiol was tested in the same way with yet another extract. A loss of 1 to 4 points of each scale reading only was observed. But estriol similarly tested was almost totally quenched. In order to find out whether these differences were due to individual characteristics of the estrogens, all three were tested against fixed amounts of the same urinary extract.

The results of these experiments showed that estrone, estradiol and estriol were only slightly quenched and all approximately to the same degree. Thus it was tentatively concluded that quenching is a characteristic of individual urinary extracts operating more or less equally on all three estrogens but varying from one extract to another to an unknown degree.

This discovery came late in the investigation after most of the clinical determinations had been done. The reliability of the results of these determinations therefore can not be accepted. The recovery experiments designed to show the total loss of estrogen in the course of the procedure were in fact also showing the effect of quenching. Future determinations would require that a recovery experiment accompany each assay of an unknown. The amount of estrogen added to the recovery specimen probably should be 10 mcg. rather than 20 mcg. in the case of estrone.

Clinical Determinations

During the course of the development and testing of the method determinations were done from time to time as clinical material became available. In this way it was hoped that an empirical standard would evolve relating results of chemical assay with clinical conditions. A very rough conversion factor was worked out to express the

scale reading in terms of estrogen equivalent. Engel's studies (57) indicated that non-pregnancy urines probably contained insignificant amounts of estradiol if any, that estrone is present in small amounts and that estriol in the post-ovulatory phase may exceed estrone by about six times (although not found in the pre-ovulatory phase). A ratio of 1:3:6 for estradiol, estrone and estriol was adopted. The results of several calibrations gave a mean scale reading per mcg. for estradiol of 66, for estrone of 27 and for estriol of 6. Thus the total scale reading for 10 mcg. of estrogens in the above ratio would be 201 or a scale reading of 20 per mcg. It became standard practice to take one twentieth of the final extract of 100 ml. for fluorometry. Hence the estrogen content of the 100 ml. = $20 \times \frac{1}{20} \times$ (scale reading) mcg.

Below are listed the results of estrogen assay in health and in some pathological conditions.

Diagnosis	24-hour estrogen equivalent
Male: normal	568 mcg.
"	661
"	880
Female: normal	357
"	400
"	415
"	454
"	594
"	629
ovarian agenesis, age 18	134
precocious puberty, age 8	238
metrorrhagia	305
metrorrhagia	869

Diagnosis	24-hour estrogen equivalent	
hirsutism	562	
menopausal symptoms	987	
diabetes, pregnancy (8th mo.)	3040	
adrenal hyperplasia	1804	17 K.S.= 105 mg./24 hr.
adrenal tumor	4536	17 K.S.= 464 mg./24 hr.
adrenal tumor	4536	17 K.S.= 465 mg./24 hr.

It is evident from these figures that the estrogen excretion in health as determined by the present method is approximately ten times greater than the expected levels (about 50 mcg. per diem according to Heard (55)). It is uncertain as to whether this applies to the pathological conditions as well. Certainly in the eighth month of pregnancy one would expect an excretion of more than 3 mg./ 24 hours. However this low result is partly due to using the non-pregnancy conversion factor. When almost all of the fluorescent activity is attributed to estriol as it should be in late pregnancy (55) then the equivalent is raised to 10 mg. In all other cases it seems clear that the present method measures something else in the urinary extract besides estrogen.

The coincidence in finding identical levels of urinary estrogen in two cases of adrenal tumor is even more striking when the 17-ketosteroid levels are found to be the same. The question arises as to whether some neutral steroid has escaped into the estrogen fraction during the partition procedure.

Experimental Conditions (continued)

7. Fluorescence of neutral steroids.

A preliminary experiment in which one twentieth of several neutral (17-ketosteroid) extracts were taken for fluorometry showed that they all contained more fluorescent material than could be read on the fluorometer scale. In testing the specificity of their method Finkelstein et al. (63) found slight fluorescent activity in desoxycorticosterone acetate, testosterone and progesterone but none in androsterone, androstanediol or various progesterone derivatives. Moreover it was assumed that the partition of the phenol group including estrogens from the neutral steroids was complete.

Tests of the fluorescence of various pure 17-ketosteroids known to occur in urine disclosed a surprising amount of activity, as is shown in Table 11.

TABLE 11. Fluorescence of 17-ketosteroids

metabolite	scale reading for 20 mcg.
androsterone	36
iso-androsterone	27
dehydroisoandrosterone	20
Δ^4 androstenedione-3,17	15
androstanedione-3,17	7

This is of the order of 1/20 the fluorescent activity of estrogens. But since the concentration of 17-ketosteroids in urine normally lies in the range 5 to 20 mg./24 hours an escape of only 1% from the neutral into the phenolic fraction would begin to affect estrogen readings.

In the present method it is assumed that the neutral steroids are retained in the toluene phase when the phenolic fraction is extracted with alkali. To test the completeness of this partition several urine specimens were assayed in duplicate, first for 17-ketosteroids by the method of Holtorff and Koch (44), and secondly for estrogens. The toluene phase of the estrogen method was saved, the toluene driven off under reduced pressure on a boiling water bath and the residue treated in the same way as the residue of the final ether extract of the 17-ketosteroid assay. If the same results were obtained by each method the escape of 17-ketosteroids from toluene would be disproved. Results are shown in Table 12.

TABLE 12. Measurement of 17-ketosteroids in toluene phase. (24-hour values)

specimen	17 K.S.(routine)	17 K.S.(from toluene)	loss
1	16.9 mg.	16.5 mg.	0.4 mg.
2	21.2	25.9	-
3	15.9	15.4	0.5
4	12.1	7.5	4.6
5	20.6	16.4	4.2
6	15.3	10.9	4.4
7	14.8	12.9	1.9

The actual loss in a 100 ml. aliquot ranged from o.02 mg. to 0.38 mg., the fluorescence of the latter being equivalent to that of 19 mcg. estrogen. The considerable differences in the results by the two methods may have been partly due to alteration of the neutral steroids by the intensive heating while driving off the toluene (up to 97°). The results for specimen 2. throw some doubt on the validity of the other figures.

Another approach to the problem was therefore adopted. From a 24-hour urine specimen duplicate 100 ml. portions were subject to estrogen assay. To one was added 1.5 mg. dehydroisoandrosterone immediately following hydrolysis. The fluorescence of the two estrogen extracts proved to be identical. This indicated that at least this particular 17-ketosteroid did not influence estrogen values. This line of investigation was continued by testing the effect of adding a mixture of pure 17-ketosteroids in one case and a neutral extract of urine in another.

From a single 24-hour urine speciment four 100 ml. aliquots were taken: A, B, C and D.

A was assayed for 17-ketosteroids and the unused portion of the neutral extract (about 90%) was saved.

B, C and D were assayed for estrogen and the toluene was saved and assayed for 17-ketosteroids.

To C, following hydrolysis, was added a mixture of pure 17-ketosteroids totalling 1.52 mg.

To D, following hydrolysis, was added the remainder (about 90%) of A's neutral extract.

Thus A established the 17-ketosteroid value for all and provided a source for neutral extract.

B established the unaltered estrogen value.

C showed the effect on this of an excess of pure 17-ketosteroids.

D showed the effect on this of an excess of the neutral fraction.

The mixture of pure 17-ketosteroids was made up from samples available in the laboratory and the proportions were adjusted to resemble those of normal urine:

androsterone	820 mcg.
iso-androsterone	400 mcg.
Δ^4 androstenedione -3,17	150 mcg.
androstanedione -3,17	150 mcg.
	1520 mcg.

This amount was selected so as to approximately double the amount found in A.

Results are shown in Table 13.

TABLE 13. Effect of variation in the neutral fraction on estrogen results.

100 ml. urine	B	C	D
+ pure K.S.		1.52 mg.	
+ neutral ex. cont. approx.			1.55 mg.
Estrogen value	60 mcg.	64.5 mcg.	71 mcg.

The 17-ketosteroid value for A was 1.72 mg. Assay of the toluene fraction of the estrogen method in specimens B, C and D gave 17-ketosteroid values of 1.5, 2.4 and 2.5 mg. respectively.

This experiment illustrated that approximate doubling of the 17-ketosteroid content of a 100 ml. specimen of urine has the effect of raising the estrogen value by roughly 3 mcg./mg. and that when all neutral substances are approximately doubled the increase is of the order of 7 mcg./mg. Thus the present method is shown to lack specificity for estrogens.

Bioassay

The final question in the investigation was whether the so-called estrogen extract of male and non-pregnancy urines contained any estrogen at all. Accordingly a male 24-hour urine was collected and the dried estrogen extract of one half the total volume was dissolved in ether and added to 4 ml. sesame oil. The ether was then evaporated. From the unused half of the 24-hour specimen 100 ml. was assayed by the fluorometric method.

A modification of the bioassay method of Klinefelter, Albright and Griswold (68) for Follicle Stimulating Hormone was used. Comparison of the extraction procedure for estrogen and FSH showed that they were mutually exclusive.

Three pairs of mice were used for standardization with estrone. The mice received 5 injections over three days and were sacrificed and the uterus examined on the fourth day. The first pair who received a total dose of 0.6 mcg./mouse showed a positive result, the second pair who received 0.2 mcg./mouse were just stimulated while the third pair who received 0.02 mcg./mouse showed no response. Thus 1 mouse unit of estrone equalled 0.2 mcg.

Four pairs of mice were selected for the test. The first pair given sesame oil alone showed no response. The second pair, each receiving 1/4 of the extract in oil, were positive as were the third pair, each receiving 1/12 of the extract. The fourth pair, each receiving 1/36 of the extract were just stimulated. Since only half the 24-hour volume had been extracted 1/72 of the total volume would contain the equivalent of 0.2 mcg. estrone. Thus the estrogen content was 72 x 0.2 mcg. estrone = 14.4 mcg. estrone /24-hours.

The result of the fluorometric assay on 100 ml. of the same urine gave an estrogen content of 582 mcg./24-hours. Even supposing a 200% error in bioassy in the direction of under-estimation the fluorometric value would still be well over ten times the bioassy value.

Discussion

It is evident from the results of the investigations described that the proposed method of estrogen estimation is unsatisfactory.

The chief fault is the gross over-estimation of estrogen as shown particularly by bioassay and supported by clinical determinations. This must be due to the presence of material in urine which resembles the estrogens in solubility and so accompanies them in the solvent partition procedure. That it also occurs in the neutral fraction and escapes into the alkali (phenolic fraction) has been shown.

A fundamental principle of the fluorometric method is that the magnitude of fluorescent activity is so great that extreme dilution of the solution is possible. In this way, theoretically, interfering background material is reduced to insignificant levels. In the present method two factors have restricted the degree of dilution. It has been shown (67) that treatment of estrogens with phosphoric acid produces only about one tenth of fluorescence that sulfuric acid treatment yields. Also the use of the stabilizer reduces sensitivity by about one third. This overall reduction in sensitivity appeared to be an advantage initially in that it allowed the same dilution of a wide variety of urinary extracts to be read on the instrument scale. However if it were possible to obtain

pure extracts sensitivity would have to be increased. This could be done by changing to sulfuric acid and eliminating the voltage stabilizer. The latter would require, in turn, that a balanced photocell circuit instrument be used to compensate for voltage variation. A continuous spectrum source or one with a more appropriate exciting wave length would also increase sensitivity.

Regarding the other findings, reproducibility was satisfactory. The variations in recovery, in quenching and in the fluorescence of pure estrogen standards could be largely overcome by carrying out a recovery concomitantly with each unknown.

But the main objection remains and applies equally to the sulfuric acid procedures. Engel (69) using counter-current distribution methods was able to demonstrate the presence of estrone and estriol in female non-pregnancy urine but this accounted for only a part of the total fluorescence. In male urine the fluorescence was nearly completely non-specific. Finkelstein (70) in an attempt to overcome the effect of background material proposed measuring its fluorescence as excited at a shorter wave length and subtracting the value obtained from the estrogen reading, but he admitted that in spite of this correction the fluorometric estrogen values were many times greater than the bioassy values. This correc-

tion was tried in the present method but the alteration of results was so slight as to be thought not worth while.

Migeon (71) in applying the counter-current distribution technique to estrogen extracts prior to fluorometry found that only 19 to 41% of the total fluorescence in various specimens could be attributed to the three estrogens.

Thus the problem is one of purification of the estrogen extract. It may be that the interfering substances in plasma are more easily dealt with as indicated by Veldhuis (72). Liquid chromatography for urinary extracts has been tried by several workers but the impurities behave so much like estriol that they accompany it on the column and on elution. Garst and Friedgood (73) described their attempted separation and identification of the background material and gave some presumptive evidence that it was made up largely of estrogen metabolites. Subsequently Garst (74) fractionated this material and obtained one fraction (about 40% of the total) which was weakly estrogenic. It resembled estriol in solubility but differed in melting-point. Its ultra-violet spectrum, different from that of estriol, was strikingly similar to that of a synthetic estrone oxidation product. Thus the possibility that much of the interfering fluorescence is due to estrogen metabolites is a real one. But until this is proven a more conservative designation for the results of fluorom-

etric assay of impure estrogen extracts is required. The terminology of Nathanson, Engel and Kelley (75) who use "Fluorogenic Phenols" is reasonably appropriate.

APPENDIX B

SUPPLEMENTARY SERIES

17 - ketosteroid values

Males

Acne	Normal
13.1 mg./24 hrs.	12.1 mg./24 hrs
18.9	
14.9	

Females

Acne	Normal
14.8 mg./24 hrs.	19.5 mg./24 hrs
16.6	12.3
23.2	7.4
18.8	15.5

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